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Hypophosphatemic rickets accompanying McCune–Albright syndrome: evidence that a humoral factor causes hypophosphatemia

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Abstract McCune–Albright syndrome (MAS) is sometimes complicated by hypophosphatemia. However, it remains unclear whether a humoral factor is associated with the cause of hypophosphatemia. We isolated cells with mutations of the Gsα gene from fibrous bone dysplasia tissues of two MAS patients (MAS cells). Severe combined immunodeficiency (SCID) mice were subjected to experiments using from one of these cells patients. Effects of conditioned media (CM) isolated from MAS cells (MAS-CM) on phosphate transport were investigated by using rat renal slices, the renal cell line OK-B, rat intestinal rings and the human intestinal cell line Caco-2. In addition, the effects of MAS-CM on human sodium-dependent phosphate transporter (*NPT2*) gene promoter activity expression were investigated in the renal cell line OK-B2400 and were compared with the effects of CM isolated from a patient with oncogenic hypophosphatemic osteomalacia (OHO). MAS cells caused significant hypophosphatemia ($P < 0.05$) and elevated serum alkaline phosphatase activity ($P < 0.05$) in SCID mice. The MAS-CM significantly inhibited phosphate uptake in everted intestinal rings ($P < 0.01$), whereas it had no effect on glucose uptake. The MAS-CM had no effect on either phosphate uptake in the kidney or *NPT2* gene promoter activity. In contrast, the CM of the OHO patient significantly inhibited phosphate uptake and *NPT2* gene promoter activity. These results indicate that the humoral factor derived from fibrous dysplasia cells of the MAS patient is different to that from OHO patients, because the humoral factor from the MAS patient inhibited phosphate transport not in the kidney but in the intestine.

Key words hypophosphatemia · osteomalacia · SCID mice · humoral factor · Gsα protein

Introduction

McCune–Albright syndrome (MAS) is characterized by café au lait spots, polyostotic fibrous bone dysplasia, and multiple endocrine hyperfunctions, such as precocious puberty, hyperthyroidism, autonomous adrenal hyperplasia and growth hormone-secreting pituitary adenoma [1]. Hypophosphatemic rickets is sometimes observed as a complication of MAS [2]. MAS is reported to be caused by activating mutations of the Gsα protein [3,4]. The reduction in guanosine triphosphatase (GTPase) activity caused by this mutation results in an increase in cyclic adenosine 3′,5′-monophosphate (cAMP) levels in the endocrine organs, which, in turn, is linked to the activation of Gsα protein-associated hormone actions [5], including increased interleukin (IL)-6 synthesis in fibrous cells isolated from affected bones [6].

Two hypotheses have been put forward to explain the pathogenesis of hypophosphatemic rickets associated with MAS. One concerns the hyperfunction of parathyroid hormone (PTH) in the renal tubules due to mutations of the Gsα protein [7] caused by the inhibitory effect of PTH via cAMP on phosphate transport in the kidney [8]. The other hypothesis is based on humoral factors from the bone lesions inhibiting phosphate transport, as proposed by Dent and Gertner [9], who presented a case report of a MAS patient whose serum phosphate level recovered after the excision of polyostotic bone lesions. It is well known that humoral factors are associated with hypophosphatemia in patients with mesenchymal benign tumors [10,11] and in Hyp mice [12], a murine model of human X-linked hypophosphatemic vitamin D-resistant rickets (XLH) [13]. Recently, a new phosphate-regulating hormone, known as phosphatonin, has been proposed as a factor involved in tumor-induced hypoposphatemic rickets [14] and in XLH [15] due to phosphate-regulating neutral endopeptidase on X chromosome (PHEX) mutations [16].

In the present study, we examined whether a humoral factor existed as a cause of hypophosphatemia using severe combined immunodeficiency (SCID) mice bearing cells isolated from the bone lesions of a MAS pa-

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tient (patient A). In addition, we assessed the effects of conditioned media (CM) prepared from the cells of another MAS patient (patient B) on phosphate uptake using rat renal slices and intestinal rings. Moreover, we studied the effect of these CM on the gene expression of the human type II sodium-dependent phosphate cotransporter NPT2 in renal cells in culture. The results of these studies indicated the presence of a humoral factor, affecting phosphate transport elsewhere than in the kidney, to account for the pathogenesis of hypophosphatemic rickets associated with MAS.

Materials and methods

Subjects

Patient A was female with café au lait spots, precocious puberty, polyostotic fibrous bone dysplasia and subclinical hyperthyroidism, as reported by us previously [6]. She was born on October 31, 1979. On admission, at the age of 1 year and 3 months, serum phosphate levels were normal. At 3 and 4 years of age, no hypophosphatemia was observed and the serum 1,25-dihydroxyvitamin D_3 ($1,25(OH)_2D$) concentration was increased slightly. The serum phosphate concentration decreased naturally from 5.2 to 4.0 mg/dl with age. However, hypophosphatemia appeared at the age of 13 years, and the results of laboratory tests performed at that time are shown in Table 1. Since her last examination in 1993, the patient has been treated with phosphate, followed by the combined administration of phosphate and 2 μg/day 1α-hydroxyvitamin D_3 ($1\alpha OHD_3$). When the patient was 15 years of age, a bone biopsy was performed at the Osaka University Hospital. Then, fibrous cells were isolated from bone fragments. Histology of an iliac bone biopsy revealed an increase in the osteoid surface and thickness, suggesting osteomalacia (Fig. 1). However, there were no hypomineralized periosteocytic bone lesions, which are a hallmark of XLH [17].

Patient B was born on December 20, 1971. The patient was female, diagnosed as having MAS when she was 2 years of age. She showed skin pigmentation, polyostotic fibrous bone dysplasia, and precocious puberty. When she was 9 years of age, hypophosphatemia appeared and, at the age of 22 years, an operation to correct her bone deformity was performed at the Osaka University Hospital. Then, fibrous cells were isolated from the bone fragments and stored at −80°C. Two years later, in 1995, the patient was again admitted to our hospital for detailed examination. Before admission, she was not taking any medication. Laboratory data at admission are shown in Table 1. Since her last examination in 1996, the patient has been treated with phosphate followed by the combined administration of phosphate and 4 μg/day $1\alpha OHD_3$. The study protocols were approved by a review board of Osaka University Hospital and informed consent was obtained from the patient.

Assays

Serum and urinary constituents were measured using standard techniques at the Osaka University Hospital

Table 1. Laboratory data for McCune–Albright syndrome (MAS) patients on admission

	MAS		
	Patient A	Patient B	Normal values
Age (years)	13	24	
Sex	Female	Female	
Serum parameters			
Ca (mg/dl)	9.20	8.4	8.4–10.0
Pi (mg/dl)	3.2		3.6–5.8
		2.5	2.6–4.3
ALP (IU/l)	2686	2143	69–185
25OHD (ng/ml)	10.7	13	9–29
$1,25(OH)_2D$ (pg/ml)	17.1		40–80
		8.4	20–35
High sensitive PTH (pg/ml)	362		160–520
Intact PTH (pg/ml)		28.6	10–65
Osteocalcin (ng/ml)	80	140	2.5–13
Urinary parameters			
Tmp/GFR (mg/dl)	3.1	2.5	2.3–4.3
Deoxypyridinoline (μmol/mmol Cr)	150	140	7.0
Nephrogenous cAMP (nmol/100 ml GFR)	1.77	0.88	0.92–2.20

Ca, calcium; Pi, phosphate; ALP, alkaline phosphatase; 25OHD, 25-hydroxyvitamin D_3; $1,25(OH)_2D$, 1,25-dihydroxyvitamin D_3; PTH, parathyroid hormone; Tmp/GFR, maximal tubular reabsorption of phosphate/glomerular filtration rate; Cr, creatinine

and Osaka National Hospital. Maximal tubular reabsorption of phosphate (Tmp)/glomerular filtration rate (GFR) values were calculated by means of Walton and Bijvoet's nomogram [18]. Serum PTH concentrations were assayed using a high-sensitivity radioimmunoassay (RIA) kit that recognizes the midportion of PTH (Yamasa Shoyu, Chosi, Japan). Serum intact PTH concentrations were measured by an immunoradiometric assay (Nichols Institute Diagnostics, San Juan Capistrano, CA, USA). Plasma and urinary cAMP concentrations were measured by RIA (Yamasa Shoyu). Serum $1,25(OH)_2D$ and 25OHD 25 hydroxyvitamin D concentrations were measured with a radioreceptor assay and a competitive protein binding assay, respectively [19]. Urinary pyridinoline and deoxypyridinoline were measured using a high-performance liquid chromatography method (Mitsubishi Kagaku BCL, Ibaraki, Japan) [20].

Bone biopsy

Bone specimens were fixed in 70% ethanol for 3 weeks. After Villanueva bone staining, the bone sections (40 μm) were ground.

Materials

Dulbecco's modified Eagle's medium (DMEM) and 199 medium were obtained from Gibco Laboratories (Grand Island, NY, USA) and Nissui Pharmaceutical (Tokyo, Japan), respectively. Collagenase, DNase type I and hyaluronidase type I-S were obtained from Sigma Immunological (St. Louis, MO, USA). Collagenase type II was purchased from Worthington Biochemical (Freehold, NJ, USA). Wistar strain rats were obtained from Japan SLC (Shizuoka, Japan). Eight-week-old SCID mice were obtained from Clea Japan (Tokyo, Japan). [^{14}C] Glucose and [^{32}P]orthophosphate were purchased from Amersham Pharmacia (Buckinghamshire, UK). Caco-2 cells were purchased from ATCC (Rockville, MD, USA). Cloned opossum kidney cells (OK-B cells) [21] were provided by Dr. J. A. Cole (University of Missouri). Intracellular cAMP content in Caco-2 cells was assayed using the Biotrak cAMP enzyme immunoassay system (Amersham). Protein was measured using a BCA kit (Pierce, Rockford, USA). K-PHOS tablets were purchased from Beach (Tampa, FL, USA).

Cell culture for fibrous cells

Bone fragments from two patients with MAS and two normal controls were digested by collagenase (2 mg/ml) and DNase I (0.1 mg/ml) in phosphate-buffered saline (PBS) for 2 h at 37°C. Isolated cells were washed twice with DMEM supplemented with 10% fetal calf serum (FCS) and then cultured as described elsewhere [6]. Characterization of cells from the MAS patients revealed low alkaline phosphatase activity and no osteocalcin synthesis by 10^{-8}M $1,25(OH)_2D$ in the culture media (data not shown). For normal controls, bone fragments from patients without any metabolic bone diseases were obtained at operation with the informed consent of the patients' parents. Cells were obtained using the same methods as for obtaining cells from the tule MAS patients. CM using DMEM supplemented with 10% FCS were obtained from confluent cells in culture, the passage numbers of which were within five for both the MAS patient B (MAS medium) and normal control. CM was collected after 48 h incubation with the cells and was stored at −80°C before the experiments.

Cell culture for the tumor cells (hemangiopericytoma) causing oncogenic osteomalacia

Tumor tissue (2 g) was removed from a patient with hemangiopericytoma at initial operation and was minced into 1-mm pieces [10]. The tissue was suspended in calcium-supplemented (1 mM) Hanks' balanced salt solution with 3 mg/ml collagenase type II and 4 mg/ml hyaluronidase type I-S, and agitated in a metabolic shaker for 11 min. Approximately 1×10^5 cells in 199 medium containing 10% FCS were plated into culture dishes. CM using 199 medium containing 10% FCS was removed on day 3 and stored at −80°C.

Mutation analysis of Gsα protein

DNA was isolated from the fibrous cells of the two MAS patients using standard methods. The DNA fragment encoding exon 8 was amplified by polymerase chain reaction (PCR) with primers described elsewhere [6], except for the deletion of the GC repeats. The DNA sequence of the PCR products was analyzed using the automated fluorescent method, as described previously [6].

Transplantation study

A total of 1×10^5 fibrous cells isolated from MAS patient A was injected subcutaneously into 8-week-old SCID mice, and blood and urinary parameters were determined after 2 months. Blood calcium and phosphate levels, and ALP activity were measured with standard techniques developed by Mitsubishi Kagaku BCL and by using an autoanalyzer. Urinary phosphate and creatinine levels were also measured with the same method as described above. The femur was fixed in 70% ethanol, prestained with Villanueva bone stain, and embedded in methyl methacrylate without decalcification. Serial sections were cut longitudinally using a

microtome (model 2050; Reichert Jung, Buffalo, NY, USA), and 7-μm sections were further stained with the Villaneueva Goldner stain for discrimination between mineralized and unmineralized bone [22].

Experiments using everted jejunal rings

Transport studies were performed using in vitro preparations of the jejunum from Wistar rats weighing 200–250 g. Twelve-hour fasted animals were killed, and the mid-jejunum was identified 10 cm beyond the duodenojejunum flexor. A 20–30-cm segment of jejunum was quickly excised, everted over a plastic rod and rinsed in chilled, oxygenated Krebs'–Henseleit phosphate buffer [23]. Everted jejunum rings from two to four rats were pooled and randomized to minimize variability between animals. Up to 10 rings were incubated at 37°C for 30 min in 25-ml Erlenmeyer flasks containing 5 ml oxygenated (100% O_2) Krebs'–Henseleit phosphate buffer in the presence or absence of the CM according to well-established methods [24]. Tissues were preincubated with 10% or 50% CM obtained from normal or MAS (patient B) cells 30 min before the transport assay. The substrates (glucose and phosphate) were then added to the incubation media as a mixture of a radioactive tracer and unlabeled substance. On average, the media contained 1.85×10^4 Bq/ml for [^{14}C]glucose and 14.8×10^4 Bq/ml for [^{32}P]orthophosphate. Measurements of phosphate and glucose uptake were performed over a 120-s period.

Effect of heat treatment of MAS medium on phosphate uptake in Caco-2 cells in culture

Caco-2 cells were grown in DMEM (glucose content 25 mmol/l) at 37°C in a 10% CO_2–90% O_2 atmosphere [25]. Monolayers of Caco-2 cells were grown on plastic petri dishes in 3 ml supplemented DMEM. Uptake studies were performed using subconfluent monolayers. Measurements of phosphate uptake were performed over a 30-min period. All uptake measurements were performed as described elsewhere [25]. To investigate heat instability, MAS medium was treated at 50°C for 2 min in a water bath.

Experiments using rat renal slices

Three to four cortical slices were incubated at 37°C for 30 min in the presence or absence of 10% or 50% MAS medium. Uptake measurements were performed as described earlier for intestinal rings.

Phosphate uptake in OK-B cells

The OK-B cells were maintained in DMEM/Ham's F-12 medium (1:1) supplemented with 10% FCS in a humidified atmosphere of 5% CO_2–95% air at 37°C. Subconfluent monolayers (4 days in culture) were used for uptake studies. Measurements of phosphate uptake were performed over a 40-min period. All uptake measurements were performed as described elsewhere [25].

Assay of NPT2 *gene promoter activity*

The luciferase reporter vector (p3P2400) containing the 5′-flanking region of the human *NPT2* gene described previously [26] was used. pCI-neo encodes geneticin (G418) resistance under an SV-40 promoter. We constructed the vector pCI-neo-p3P2400 (pCI-24), which contains cDNA encoding luciferase under the *NPT2* gene promoter. After transfection of pCI-24 to OK-B cells, colonies of geneticin-resistant cells (termed OK-B2400 cells) were isolated and analyzed for the expression of luciferase. These cells were plated with DMEM/F12 (1:1) containing 10% FBS. After 2 days, the medium was changed to the assay medium with 50% MAS medium. For the analysis of luciferase activity, cells were lysed with a cell lysis buffer containing 1% Triton X-100 and then centrifuged. The supernatant was analyzed for luciferase activity, which was measured by a luminometer [25,27]. Protein concentrations were determined with a BCA protein assay reagent.

Statistical analysis

The results are shown as the mean ± SD and data were analyzed using Student's *t*-test or ANOVA (Stat View J 5.0; SAS, Cary, NC, USA).

Results

Laboratory data on admission

Serum calcium levels were normal in MAS patient A, and decreased in MAS patient B. In both patients, serum phosphate levels were slightly decreased or toward the lower range of normal, and serum alkaline phosphatase (ALP) activity was elevated. In these patients, serum 25OHD and PTH levels were normal. Serum $1,25(OH)_2D$ levels were low in the presence of slightly decreased serum phosphate levels. Maximal tubular reabsorption of phosphate (Tmp/GFR) and nephrogenous AMP values were within the normal range. Urinary deoxypyridinoline levels were markedly increased in both cases (Table 1).

Mutation analysis of Gsα protein

Cells isolated from the fibrous bone dysplasia tissues of the two MAS patients revealed a point mutation of Arg^{201} to His in MAS patient A [6] and Arg^{201} to Cys in MAS patient B. DNA sequence analysis showed that

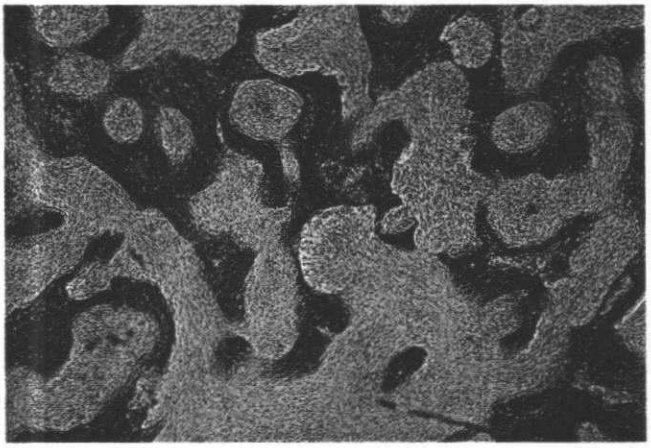

Fig. 1. Histology of iliac bone biopsy of McCune–Albright syndrome (MAS) patient A. The bone specimen was stained with Villanueva bone stain. Note increased osteoid tissues (*green*) on bone surfaces and growth of fibroblast-like cells in the bone marrow space (×40)

cells isolated from patients A and B contained mutated and normal Gsα genes, respectively (data not shown).

Transplantation study

Table 2 shows data from experiments with SCID mice. In SCID mice bearing cells from MAS patient A, serum phosphate levels were significantly reduced, while serum ALP levels were significantly increased compared with those of sham-operated animals (Table 2). In addition, in the same experimental series, histological examination showed that the transplanted MAS (patient A) cells caused a marked increase in osteoid tissue in the femur of SCID mice (Fig. 2). Preliminary bone histomorphometry measurements revealed that osteoid tissues in the secondary ossification center (osteoid surface/bone surface) were significantly increased in SCID mice bearing MAS cells compared with SCID mice bearing normal control cells.

Effects of MAS medium on phosphate uptake in rat jejunal rings

Data for phosphate uptake in the rat jejunum are shown in Fig. 3. Phosphate uptake was significantly inhibited by 50% MAS medium. In contrast, the medium had no effect on glucose uptake. At 10%, MAS medium inhibited neither phosphate nor glucose uptake (data not shown).

Effect of heat treatment of MAS medium on phosphate uptake in Caco-2 cells

The uptake of phosphate was analyzed in monolayers of the established intestinal cell line Caco-2. In the presence of sodium, linear uptake of phosphate was observed between 3 and 40 min (data not shown). After incubation of Caco-2 cells with 50% MAS medium, phosphate transport activity was markedly inhibited. In contrast, heat treatment of the MAS medium at 50°C for 2 min completely abolished the inhibitory effect of the MAS medium on phosphate uptake in Caco-2 cells in culture (Fig. 4).

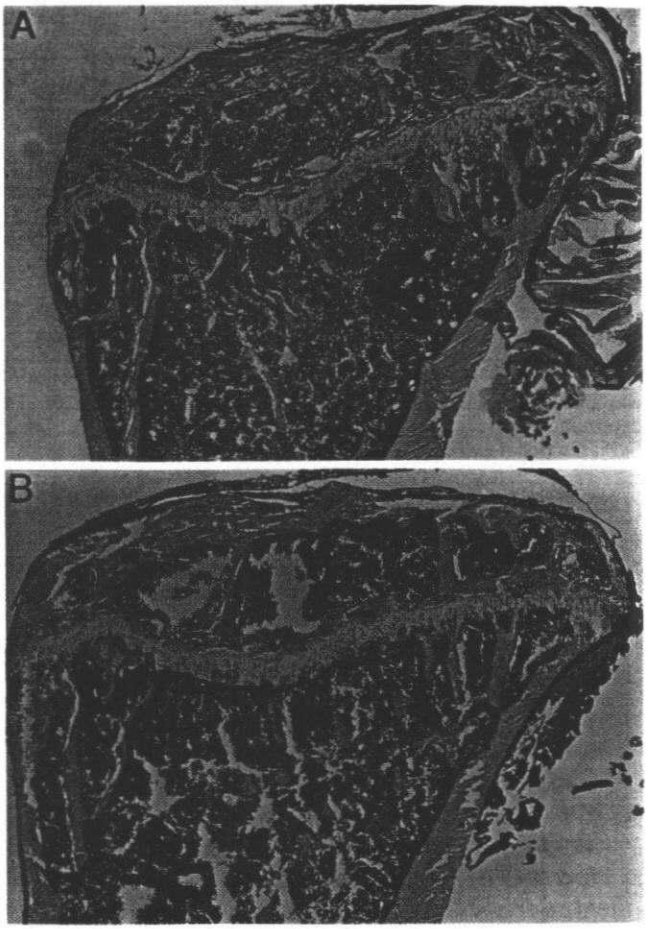

Fig. 2. Histology of the femur of the severe combined immunodeficiency (SCID) mice bearing cells from the MAS patient A (**A**) and a normal control (**B**). Bone specimens were stained with Villanueva Goldner stain. Note increased osteoid tissues on the bone surfaces (*red*) of the secondary ossification center (×40)

Effect of MAS medium on phosphate uptake in rat kidney slices

To investigate the effects of MAS medium on renal phosphate uptake, we measured phosphate transport activity in renal cortical slices. In the presence of sodium, linear uptake of phosphate was observed between 10 s and 4 min (data not shown). Three to four slices were incubated at 37°C for 30 min in the presence of

Table 2. Blood and urinary parameters in severe combined immunodeficiency (SCID) mice bearing MAS patient cells

	MAS (n = 2)	Control (n = 3)	P values
Blood parameters			
Ca (mg/dl)	11.3 ± 0.919	12.3 ± 0.529	NS
Pi (mg/dl)	10.7 ± 0.849	13.3 ± 0.462	<0.05
ALP (IU/l)	188 ± 7.07	143 ± 11.7	<0.05
Cr (mg/dl)	0.400 ± 0.141	0.433 ± 0.058	NS
Urinary parameters			
FePi	18.5 ± 1.41	25.6 ± 2.88	NS
FePi/Pi × 10	17.3 ± 0.049	19.4 ± 2.71	NS

A total of 1×10^5 fibrous cells from MAS patient A (n = 2) and normal control (n = 3) per mouse was injected subcutaneously into SCID mice. After 2 months, blood and urinary parameters were determined. Statistical analysis was performed with Student's t-test
FePi, fractional excretion of phosphate

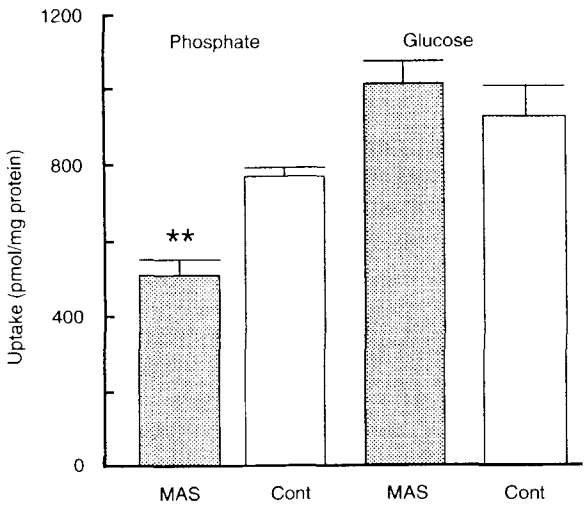

Fig. 3. Effects of the MAS medium prepared from MAS patient B on phosphate and glucose uptake in rat jejunal rings. Transport studies used everted jejunum rings pretreated with 50% MAS media or the same concentration of normal conditioned medium (CM). After incubation with MAS medium, jejunal rings were washed with Krebs'–Henseleit phosphate buffer. The transport assay was determined for 30 min in 0.1 mM substrate (KH_2PO_4 or D-glucose). Data are quadriplicate determinations. $^{**}P < 0.01$ compared with control (*Cont*)

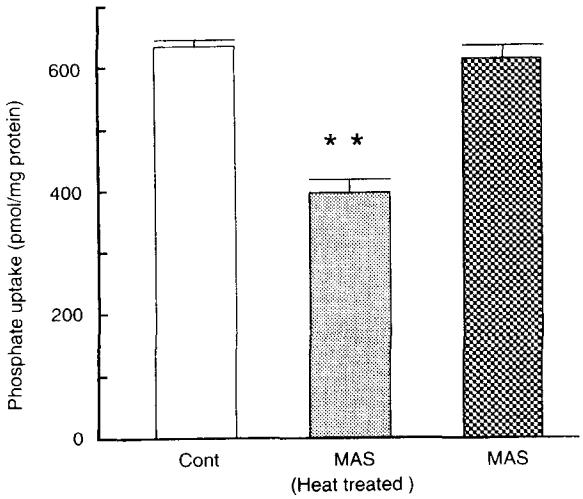

Fig. 4. Effects of heat treatment on phosphate uptake in Caco-2 cells in culture. Monolayers of Caco-2 were preincubated with culture medium containing 50% MAS medium prepared from MAS patient B or the same concentration of normal CM for 8 h. Heat treatment was performed at 50°C for 2 min. Data are quadruplicate determinations. $^{**}P < 0.001$ compared with MAS medium without heat treatment

50% MAS medium and phosphate uptake was then measured at 2 min. As shown in Fig. 5, 50% MAS medium inhibited neither phosphate nor glucose uptake in renal slices.

Characterization of the humoral factors using OK-B and OK-B2400 cells

Phosphate uptake in OK-B cells. We investigated whether humoral factors in the MAS medium are identical to the putative humoral factor phosphatonin in oncogenic osteomalacia (OHO). In a previous study, a tumor with the histological characteristics of a hemangiopericytoma was transplanted to athymic nude mice [10]. The tumor-bearing mice had hypophosphatemia, high serum ALP levels, and increased urinary phosphate excretion. In the present study, we investigated the inhibition of phosphate transport in the primary culture medium of the tumor cells.

OK-B cells exhibit high levels of Na^+-dependent phosphate, glucose and L-leucine uptake. Phosphate uptake in the absence of extracellular Na^+ was less than 10% of total uptake in the presence of Na, and the uptake was linear for the first 10 min (data not shown). In OK-B cells, the culture medium derived from

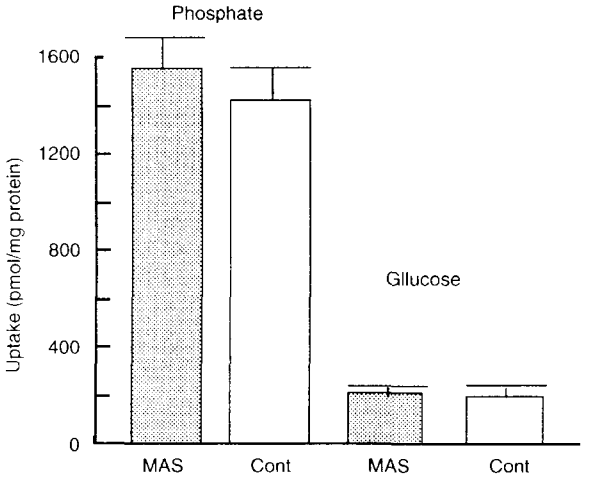

Fig. 5. Effects of MAS medium prepared from MAS patient B on phosphate and glucose uptake in rat renal slices. Transport studies were performed using the same methods as for Fig. 3. Data are quadruplicate determinations

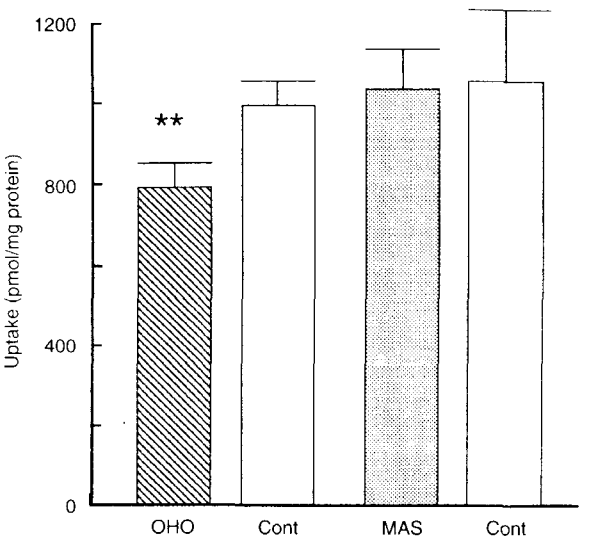

Fig. 6. Effect of MAS medium prepared from MAS patient B and oncogenic hypophosphatemic osteomalacia (OHO) medium on phosphate uptake in OK-B cells. Cells were preincubated with 50% MAS or the same concentration of OHO medium for 8 h. Data are quadruplicate determinations. $^{**}P < 0.01$ compared with control

hemangioperocytoma (OHO medium; 50%) significantly inhibited phosphate uptake. No significant inhibition was shown in OK-B cells that were preincubated with 50% MAS medium (Fig. 6).

Effect of MAS medium on NPT2 *gene promoter activity.* To study the effects of MAS medium on the transcriptional activity of human *NPT2* gene promoter, we measured luciferase activity in OK-B2400 cells that stably express the luciferase gene containing the human *NPT2* gene promoter. As shown in Fig. 7, high luciferase activity was found in OK-B2400 cells. In contrast, luciferase activity was not detected in non-transfected OK-B cells. The OHO medium significantly decreased luciferase activity compared with control medium. In contrast, MAS medium had no effect on luciferase activity.

Discussion

Hypophosphatemic rickets has been reported as a complication of MAS [2], and two hypotheses have been put forward to account for the cause of hypophosphatemia. One concerns the hyperfunction of PTH in the renal tubules. This hypothesis is based on a point mutation of the Gsα protein observed in the kidneys of MAS patients [3], which could enhance the synthesis of cAMP in the renal proximal tubules. In turn, cAMP is known to inhibit phosphate reabsorption in the kidney [8]. In fact, Zung et al. [7] have reported elevated urinary cAMP levels in several MAS patients. Thus, it seems plausible that hyperfunction of PTH in the kidney is a cause of hypophosphatemia in MAS patients. However, nephrogenous cAMP values were normal in the two MAS patients in the present study, suggesting that hyperfunction of PTH due to a point mutation in the Gsα protein in the kidney cannot explain the pathogenesis of hypophosphatemia in these patients. This apparent contradiction can be interpreted as the result of differences in the frequency of mutations in the Gsα protein in the kidney, because this occurs in MAS patients in the post-zygotic stages [28].

The second hypothesis proposed to account for hypophosphatemia in MAS suggests that a humoral factor derived from bone lesions causes hypophosphatemia [9]. To obtain evidence for the secretion of a humoral factor from cells isolated from fibrous dysplasia bone lesions, we used SCID mice bearing subcutaneously injected fibrous cells that had been isolated from bone lesions of MAS patient A. Our experiment is the first to show that the transplantation of fibrous cells from a MAS patient into SCID mice leads to a reduction in serum phosphate and an increase in serum ALP concentrations 8 weeks after transplantation. These data support the hypothesis of the involvement of a humoral factor in the pathogenesis of hypophosphatemia accompanying MAS.

The target tissue of the humoral factor affecting phosphate transport in MAS patients has been assumed to be the renal proximal tubules, as is the case for the substance known as phosphatonin in tumors induced by hypophosphatemic rickets [14] and XLH [15] due to PHEX mutations [16]. Urinary phosphate excretion

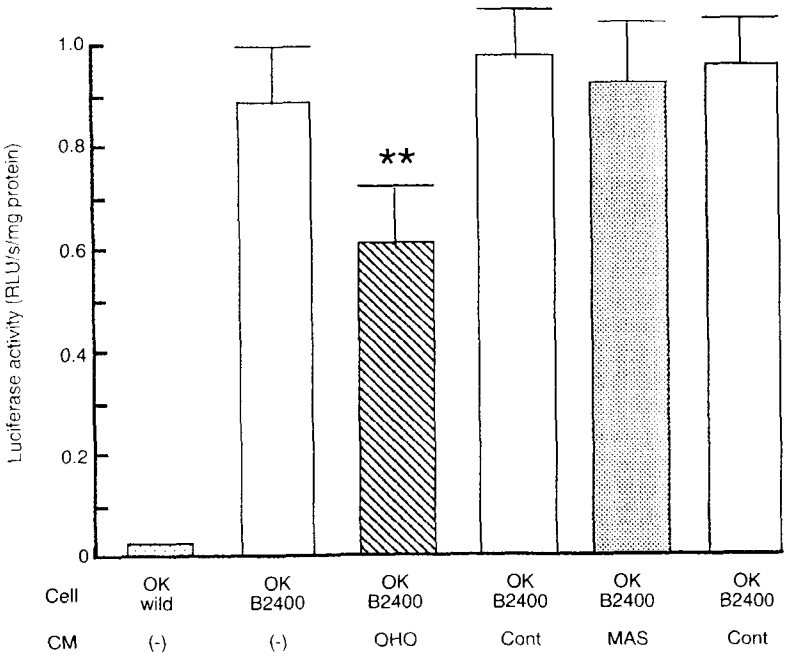

Fig. 7. Effect of the culture medium on *NPT2* gene promoter activity. Monolayers of OK-B2400 cells with *NPT2* gene were preincubated for 8 h with 50% MAS medium prepared from MAS patient B or the same concentration of OHO medium. Data are quadruplicate determinations. $^{**}P < 0.01$ compared with control. *Wild*, wild-type cells; *CM*, conditioned medium; *RLU*, relative light unit

was slightly reduced in SCID mice bearing MAS cells. However, this observation is speculated to be due to the different serum phosphate concentrations in SCID mice bearing MAS cells and normal cells, because urinary phosphate excretion values corrected by serum phosphate concentrations showed no difference between the two groups of mice. Thus, it is unlikely that the mechanism of hypophosphatemia associated with the humoral factor in MAS patients is related to a renal phosphate leak.

The intestine is known as an organ that is associated with the regulation of serum phosphate levels, although it does not regulate these levels as precisely as does the kidney [29]. However, we recently reported a case of hypophosphatemic rickets with intestinal phosphate loss caused by congenital microvillous atrophy [30]. Thus, it is plausible that the intestine is the target site for this postulated novel humoral factor, which would account for the formation of hypophosphatemic rickets in MAS patients.

To further examine our hypothesis, we investigated the effects of CM isolated from the fibrous cells of MAS patient B with hypophosphatemia on phosphate transport in the kidney and intestine. Our experiments revealed that it is plausible that the humoral factor in our MAS patient is a new factor that does not inhibit phosphate transport in the kidney but does affect phosphate transport in the small intestine. We then characterized this factor by using renal proximal tubular cells that consistently express the *NPT2* gene promoter upstream of the luciferase gene (OK-B2400 cells). The results indicated that the factor in the MAS medium is different from phosphatonin, because this new factor had no effect on the expression of the *NPT2* gene, while the culture media isolated from a patient with OHO markedly reduced *NPT2* gene expression. However, this new factor is similar to phosphatonin in that the new factor did not affect the cAMP content of the rat jejunum (data not shown). It is speculated that this factor is a peptide because the inhibitory effect on phosphate transport in the intestine was lost when the MAS medium was heated.

The relationship between this new humoral factor and Gsα protein mutation in MAS patients remains unclear; however, one of the possible explanations for the mechanism of hypophosphatemia is the action of a cytokine that is linked to a Gsα protein mutation, such as interleukin (IL)-6 [6]. However, IL-6 had no effect on phosphate transport in the jejunal rings (data not shown). Other cytokines, such as IL-1α and tumor necrosis factor-α, are reportedly associated with the causes of hypophosphatemia in humans [31]. However, these cytokines are unlikely to be candidates for the humoral factor derived from bone lesions because the serum levels of these cytokines were undetectable in our patient (data not shown).

Recently, a major sodium–phosphate cotransporter in the intestine, named type II, has been reported [32]. In addition, fibroblast growth factor 23 has been proposed to be associated with the cause of autosomal dominant hypophosphatemic rickets [33]. Thus, the nature and functions of the humoral factors in the MAS patients in relation to these new proteins need to be clarified in future studies.

In conclusion, we are able to show the presence of a novel humoral factor in the case of MAS patients with hypophosphatemic rickets. It is plausible that this factor is different from phosphatonin because it neither increased renal phosphate excretion in experiments with SCID mice, nor did it inhibit renal transport in rat renal slices.

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References

1. Spiegel AM, Shenker A, Weinstein LS (1992) Receptor effector coupling by G protein: implication for normal and abnormal signal transduction. Endocr Rev 13:536–565
2. Les PA, Van Dop C, Migeon CJ (1986) McCune–Albright syndrome: long term follow-up. JAMA 256:2980–2984
3. Weinstein LS, Shenker A, Gejman PV, Merino MJ, Friedman E, Spiegel AM (1991) Activating mutations of the stimulatory G protein in the McCune–Albright syndrome. N Engl J Med 325: 1688–1695
4. Schwindinger WF, Francomano CA, Levine MA (1992) Identification of a mutation in the gene encoding the α subunit of the stimulatory G protein of adenylyl cyclase in McCune–Albright syndrome. Proc Natl Acad Sci USA 89:5152–5156
5. Landis CA, Masters SB, Spada A, Pace AM, Bourne HR, Vallar L (1989) GTPase inhibiting mutations activate the α chain of Gs and stimulate adenylyl cyclase in human pituitary tumors. Nature 34:692–696
6. Yamamoto T, Ozono K, Kasayama S, Yamaoka K, Kishimoto T, Okada S (1996) Increased IL-6 production by cells isolated from the fibrous bone dysplasia tissues in patients with McCune–Albright syndrome. J Clin Invest 98:30–35
7. Zung A, Chaleur SA, Schwindinger WF, Levine MA, Phillip M, Jara A, Counts DR, Kowaski AA (1995) Urinary cyclic adenosine 3′-5′monophosphate response in McCune–Albright syndrome: clinical evidence for altered renal adenylyl cyclase activity. J Clin Endocrinol Metab 80:3576–3581
8. Caverzasio J, Rizzoli R, Bonjour JP (1986) Sodium-dependent phosphate transport inhibited by parathyroid hormone and cyclic AMP stimulation in an opossum kidney cell line. J Biol Chem 261:3233–3237
9. Dent VE, Gertner JM (1976) Hypophosphataemic osteomalacia in fibrous dysplasia. Q J Med NS 179:411–420
10. Miyauchi A, Fukase M, Tsutsumi M, Fujita T (1988) Hemangiopericytoma-induced osteomalacia: tumor transplantation in nude mice causes hypophosphatemia and tumor extract inhibited renal 25-hydroxyvitamin D 1-hydroxylase activity. J Clin Endocrinol Metab 67:46–53
11. Drezner MK (1996) Tumor-induced rickets and osteomalacia. In: Favus MJ (ed.) Primer on the Metabolic Bone Diseases and Disorders of Mineral Metabolism, 3rd edn. Lippincott-Raven, Philadelphia, pp 319–325
12. Jajeunesse D, Meyer RA Jr, Hamel L (1996) Direct demonstration of a humorally-mediated inhibition of renal phosphate transport in HYP mouse. Kidney Int 50:1531–1538
13. Eicher EM, Southard JL, Scriver CR, Glorieux FH (1976) Hypophosphatemia: mouse model for human familial hypophosphatemic (vitamin D resistant) rickets. Proc Natl Acad Sci USA 73:4667–4671
14. Econs MJ, Drezner MK (1994) Tumor-induced osteomalacia: unveiling a new hormone. N Engl J Med 330:1679–1681
15. Nelson AE, Robinson BG, Manson RS (1997) Oncogenic osteomalacia: is there a new phosphate regulating hormone? Clin Endocrinol 47:635–642
16. The Hyp Consortium (1995) A gene (*PEX*) with homologies to endopeptidase is mutated in patients with X-linked hypophosphatemic rickets. Nat Genet 11:130–136
17. Frost HM (1958) Some observations on bone mineral in a case of vitamin D resistant rickets. Henry Ford Hosp Med Bull 6:300–310
18. Walton RJ, Bijvoet OLM (1975) Nomogram for derivation of renal threshold phosphate concentration. Lancet ii:309–310
19. Yamaoka K, Tanaka H, Kurose H, Shima M, Ozono K, Nakajima S, Seino Y (1989) Effect of single oral phosphate loading on vitamin D metabolites in normal subjects and X-linked hypophosphatemic rickets. Bone Miner 7:159–169
20. Black D, Dunkan A, Robin SP (1988) Quantitative analysis of the pyridimium crosslinks of collagen in urine using ion-paired reverse-phase high-performance liquid chromatography. Anal Biochem 169:197–203
21. Cole JA, Forte LR, Krause WJ, Thorne PK (1989) Clonal sublines that are morphologically and functionally distinct from parental OK cells. Am J Physiol 256:F672–F679
22. Yamato H, Nagai Y, Inoue D, Ohnishi Y, Ueyama Y, Ohno H, Matsumoto T, Ogata E, Ikeda K (1995) In vivo evidence for progressive activation of parathyroid hormone-related peptide gene transcription with tumor growth and stimulation of osteoblastic bone formation at an early stage of humoral hypercalcemia of cancer. J Bone Miner Res 10:36–44
23. Agar WT, Hird FJR, Sidhu GS (1954) The uptake of amino acid by the intestine. Biochim Biophys Acta 14:80–84
24. Taketani Y, Segawa H, Chikamori M, Tanaka K, Kido S, Yamamoto H, Inoue Y, Tatsumi S, Tsugawa N, Okano T, Kobayashi T, Miyamoto K, Takeda E (1998) Regulation of type II renal Na^+-dependent Pi transporter by $1,25(OH)_2D_3$: identification of a vitamin D-responsive element in the human *NaPi-3* gene. J Biol Chem 273:14575–14581
25. Mohrmann I, Mohrmann M, Biber J, Murer H (1986) Sodium-dependent transport of Pi by an established intestinal epithelial cell line (Caco-2). Am J Physiol 250:G323–G330
26. Taketani Y, Miyamoto K, Tanaka K, Katai K, Chikamori M, Tatumi S, Segawa H, Yamamoto H, Morita K, Takeda E (1997) Gene structure and functional analysis of the human Na^+/phosphate co-transporter (NaPi-3). Biochem J 324:927–934
27. Biber J, Forgo J, Murer H (1988) Modulation of Na^+-Pi cotransport in opossum kidney cells by extracellular phosphate. Am J Physiol 255:C155–C161
28. Happle R (1986) The McCune–Albright syndrome: a lethal gene surviving by mosaicism. Clin Genet 29:321–324
29. Broadus AE (1996) Mineral balance and homeostasis. In: Favus MJ (ed.) Primer on the Metabolic Bone Diseases and Disorders of Mineral Metabolism, 3rd edn. Lippincott-Raven, Philadelphia, pp 57–63
30. Kagitani K, Yamamoto T, Miki K, Matsumoto S, Shima M, Tajiri H, Harada T, Okada S (1998) Hypophosphatemic rickets accompanying congenital microvillous atrophy: a case report. J Bone Miner Res 13:1946–1952
31. Barak V, Schwartz A, Kalickman I, Nisman B, Gurmane G, Shoenfeld Y (1998) Prevalence of hypophosphatemia in sepsis and infection: the role of cytokine. Am J Med 104:40–47
32. Hilfiker H, Hattenhauer O, Traebert M, Forster I, Murer H, Biber J (1998) Characterization of a murine type II sodium–phosphate cotransporter expressed in mammalian small intestine. Proc Natl Acad Sci USA 95:14564–14569
33. The ADHR Consortium (2000) Autosomal dominant hypophosphatemic rickets is associated with mutations in *FGF23*. Nat Genet 26:345–348